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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/52, 15/53, 15/11, 15/82, 9/02, 9/88, 5/10, A01H 5/00	A1	(11) International Publication Number: WO 98/06852	(43) International Publication Date: 19 February 1998 (19.02.98)
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(21) International Application Number: PCT/US97/14184

(22) International Filing Date: 11 August 1997 (11.08.97)

(30) Priority Data:  
08/695,412 12 August 1996 (12.08.96) US

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.  
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE RIPENING OF COFFEE PLANTS

(57) Abstract

Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith for transforming coffee plants to suppress the expression of enzymes necessary for ethylene synthesis. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for the enzymes ACC synthase or ACC oxidase that are elements of the pathway for ethylene biosynthesis in coffee plants. Coffee plants are transformed with vectors containing ACC synthase and/or with ACC oxidase DNA sequences that code on expression for the respective mRNA that is antisense to the mRNA for ACC synthase and/or ACC oxidase. The resulting antisense mRNA binds to XMI mRNA, thereby inactivating the mRNA encoding one or more enzymes in the pathway for ethylene synthesis. The described DNA sequences can also be used to block synthesis of ACC synthase or ACC oxidase using co-suppression. The result in either event is that the transformed plants are incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.

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PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND  
PROCESSES FOR CONTROLLING THE RIPENING OF  
COFFEE PLANTS

5 FIELD OF THE INVENTION

This application relates to purified proteins,  
recombinant DNA sequences, hosts transformed  
therewith and processes for controlling the ripening  
of coffee plants. More particularly, this  
10 application relates to purified proteins, and  
recombinant DNA sequences that can be used to  
suppress the expression of coffee fruit-specific 1-  
aminocyclopropane-1-carboxylic acid (ACC) synthase  
and ACC oxidase genes. This application further  
15 relates to coffee plants transformed with such  
sequences, thereby rendered incapable of  
synthesizing ethylene necessary for ripening.  
Application of exogenous ethylene to plants  
transformed in accordance with this invention makes  
20 it possible to synchronize and control fruit  
ripening in coffee plants.

BACKGROUND OF THE INVENTION

Coffee is prepared from the roasted beans of  
the plants of the genus *Coffea*, generally from the  
25 species *C. arabica*. Beans are the seeds of the  
coffee plant and are obtained by processing the  
fruit, most ideally mature fruit which commands the

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best price due to its superior quality. In the past, high quality "gourmet" coffee was hand picked. This is necessary because the fruits of a coffee tree do not ripen uniformly and thus there are both  
5 mature and immature fruit on the same tree. In the past, this was not a serious problem as most coffee is grown in areas of the world where labor is plentiful and not expensive. However, more recently lack of abundant and inexpensive labor has become a  
10 major contributor to decreased productivity in coffee production. To increase productivity some regions of the world, such as the largest coffee producing country, Brazil, have resorted to strip harvesting where workers rapidly remove all fruit  
15 from a branch whether ripe or unripe. This increases the speed of harvesting but decreases the yield of the highest quality beans as much of the fruit is immature (green).

Furthermore, the lack of uniform ripening has  
20 seriously limited the effectiveness of mechanical harvesting. The force required to remove mature fruit (cherry) from the tree is similar to the force required to remove green fruit. Thus, mechanical harvesters do not distinguish well between green and  
25 cherry and a large amount of immature fruit is harvested along with mature fruit. This greatly

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decreases the yield of mature fruit and limits productivity. If coffee fruit ripening could be controlled so that all fruit ripened at one time, both the strip method of hand harvesting and  
5 mechanical harvesting would be much more efficient and a higher percentage of the harvested fruit would be in the higher quality grades. This would increase profitability of coffee production.

As is the case with many other fruit [Yang and  
10 Hoffman, Ann. Rev. Plant Physiol. 35:155 (1984)], plant-produced ethylene plays an important role in the final stages of fruit ripening in coffee. Once coffee fruit reach a certain stage of maturity they can be induced to ripen by the exogenous application  
15 of ethylene [Crisosto, C.H., P.C. Tausend, M.A. Nagao, L.H. Fuchigami and T.H.H. Chen, J. Haw. Pac. Agri. 3:13-17 (1991)]. This demonstrates the importance of ethylene for the final stages of fruit ripening in coffee.

20 Ethylene is synthesized in a two-step reaction from S-adenosylmethionine (SAM). The first step is the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM by ACC synthase. In most plants this is the rate limiting step. The final step is  
25 the conversion of ACC to ethylene which is catalyzed by ACC oxidase (Yang and Hoffman, *supra*).

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Inhibition of ethylene biosynthesis by chemical (e.g., silver ions or carbon dioxide) or biotechnological means [Oeller et al., Science 254:437 (1991)] inhibits the final stages of  
5 ripening. This inhibition is reversible by the application of ethylene.

Accordingly, a strategy for controlling the ripening of coffee plants is to prevent synthesis of specific enzymes in the pathway for ethylene  
10 biosynthesis. In one embodiment this invention relates to genetic alteration of coffee plants to eliminate synthesis of ACC synthase; in another, ACC oxidase synthesis is suppressed. In the presently preferred embodiments, synthesis of one or both of  
15 these enzymes is suppressed by transforming coffee plants with a DNA sequence that codes on transcription for a messenger RNA (mRNA) that is antisense to the mRNA that codes on expression for the enzyme whose synthesis is to be suppressed. See  
20 Oeller et al., Science 254:437 (1991), who reported controlling ripening of tomatoes using a similar strategy.

Recombinant DNA technology has been used to isolate a number of ACC synthase and ACC oxidase  
25 genes. However, the genes for ACC synthase and ACC

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oxidase in coffee have not been identified or sequenced to date.

#### SUMMARY OF INVENTION

5 Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith, for transforming coffee plants to suppress the expression of enzymes necessary for ethylene  
10 synthesis. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for the enzymes ACC synthase or ACC oxidase that are elements of the pathway for ethylene biosynthesis in coffee plants.

15 Coffee plants are transformed with vectors containing ACC synthase and/or with ACC oxidase DNA sequences inserted so that the transforming sequences code on expression for the respective RNA that is antisense to the mRNA for ACC synthase  
20 and/or ACC oxidase. The resulting antisense RNA binds to mRNA(s), thereby inactivating the mRNA encoding one or more enzymes in the pathway for ethylene synthesis. The described DNA sequences can also be used to block synthesis of ACC synthase or  
25 ACC oxidase using co-suppression. The result in either event is that the transformed plants are

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incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.

Ripening in the transformed plants can be regulated by exogenous ethylene. By application of  
5 ethylene to the entire plant, the entire plant will ripen at once, making mechanical harvesting of coffee more productive.

#### SUMMARY OF THE DRAWINGS

FIGURE 1 is the complete sequence of the cDNA  
10 encoding coffee fruit expressed ACC synthase.

FIGURE 2 is the amino acid sequence of the coffee fruit ACC synthase deduced from the cDNA sequence shown in FIGURE 1.

FIGURE 3 is the sequence of the cDNA encoding  
15 coffee fruit expressed ACC oxidase.

FIGURE 4 is the amino acid sequence of the coffee fruit ACC oxidase deduced from the cDNA sequence shown in FIGURE 3.

#### DETAILED DESCRIPTION OF THE INVENTION

20 In order that the invention herein described may be more fully understood, the following detailed description is set forth. In the description the following terms are employed:

Nucleotide -- A monomeric unit of DNA or RNA  
25 consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is



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linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases  
5 are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C, and uracil ("U").

DNA Sequence -- A linear array of nucleotides connected one to the other by phosphodiester bonds  
10 between the 3' and 5' carbons of adjacent pentoses.

Codon -- A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide  
15 triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal, which also encodes the amino acid methionine ("MET").

20 Polypeptide -- A linear array of amino acids connected one to the other by peptide bonds between the amino and carboxy groups of adjacent amino acids.

Genome -- The entire DNA of a cell or a virus.  
25 It includes inter alia the structural gene coding for the polypeptides of the substance, as well as

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promoter, transcription and translation initiation and termination sites.

Gene -- A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of 5 amino acids characteristic of a specific polypeptide.

Transcription -- The process of producing mRNA from a gene or DNA sequence.

Translation -- The process of producing a 10 polypeptide from mRNA.

Expression -- The process undergone by a gene or DNA sequence to produce a polypeptide. It is a combination of transcription and translation.

Plasmid -- A nonchromosomal double-stranded DNA 15 sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. 20 For example, a plasmid carrying the gene for tetracycline resistance (TETR) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

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Phage or Bacteriophage -- Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

Cloning Vehicle -- A plasmid, phage DNA, cosmid  
5 or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological  
10 function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A  
15 cloning vehicle is often called a vector.

Cloning -- The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

20 Recombinant DNA Molecule or Hybrid DNA - A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and able to be maintained in living cells..

25 cDNA - A DNA strand complementary to an mRNA that codes for a particular polypeptide.

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The strategy for controlling ethylene biosynthesis in coffee plants according to the present invention relates in the first instance to determination of the genes that code on expression  
5 for two enzymes in the ethylene pathway: ACC synthase and ACC oxidase. Transformation of wild type coffee plants with constructs containing either or both genes in an orientation that is antisense to the normal genes is expected to block synthesis of  
10 the respective enzymes. Messenger RNA transcribed under direction from the transforming sequence will bind to mRNA transcribed under direction from the normal sequence, thereby inactivating the normal message and precluding enzyme synthesis.

15 To isolate the DNA sequences that code on expression for ACC synthase and ACC oxidase in coffee, we screened a cDNA library produced from coffee plant tissue with synthetic DNA probes containing nucleotide sequences expected to occur.  
20 These expected sequences were based on studies of nucleotide sequences that occur in genes that encode the respective enzymes, other climacteric plants and other plants.

The cDNA corresponding to the gene encoding ACC  
25 synthase or ACC oxidase is used to transform embryonic coffee plants. The plasmid pBI-121 is

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used as a transforming vector. The sequences corresponding to DNA that codes on expression for ACC synthase or ACC oxidase is inserted into the plasmid in an inverted orientation adjacent to a

5 cauliflower mosaic virus 35S promoter. RNA transcribed therefrom will be complementary to mRNA that encodes the amino acid sequence of the respective enzyme. Complete constructs are amplified in bacterial hosts. The hosts are

10 disrupted and the amplified vector is attached to colloidal gold particles. The gold particles with adherent vectors are inserted into coffee plant tissue by propelling the particles at high speed at the cells as described in U.S. patent 5,107,065.

15 Young plants successfully transformed are identified by antibiotic resistance. The transformed plants do not produce ACC synthase or ACC oxidase, depending on the gene used to transform the plants. Ripening of the transformed plants is initiated by

20 application of exogenous ethylene.

**EXAMPLE 1****Isolation of Coffee Fruit-Specific ACC Synthase cDNA**

In order to isolate ACC synthase gene sequences involved in the ripening of coffee, a cDNA library

25 was prepared from a mixture of coffee fruit pericarp and mesocarp tissue at different stages of ripeness.

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This library was screened using a PCR product synthesized from first-strand cDNA made from the same mRNA used to construct the library and degenerate oligonucleotide primers corresponding to  
5 consensus sequences derived from ACC synthase genes from other organisms. This example principally involved the isolation of mRNA, the construction of a cDNA library, and the subsequent steps involved in cloning the appropriate cDNA.

10 a) Isolation of mRNA

Total RNA was isolated from 66 g of pericarp and mesocarp tissue from several different developmental stages of coffee fruit (*C. arabica* L. cv Guatemalan) using the method of Levi et al.,  
15 [Hort Science 27(12):1316-1318 (1992)]. Frozen coffee fruit pericarp and mesocarp tissue was powdered by grinding for about 2 minutes in a domestic coffee mill (Salton Model GC-5; Salton Maxam Housewares Group, Mt. Prospect, IL) with a  
20 small piece of dry ice. The powdered fruit tissue was added to 200  $\mu$ L of 200 mM tris[hydroxymethyl]aminomethane hydrochloride (tris-HCl) (pH 8.5), 1.5% sodium dodecyl sulfate (SDS), 300 mM LiCl, 10 mM disodium  
25 ethylenediaminetetraacetic acid (Na<sub>2</sub>EDTA), 1.5% sodium deoxycholate (w:v), 1.5% Nonidet P-40 (Sigma

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Chemical Co.) (v:v), 0.5 mM thiourea, 1 mM  
aurintricarboxylic acid, 10 mM dithiothreitol (DTT),  
75 mM B-mercaptoethanol, 2% polyvinylpyrrolidone  
(PVP) and 2% polyvinylpoly-pyrrolidone (PVPP) and  
5 homogenized using a Polytron tissue homogenizer  
(Tekmar, Cincinnati, OH). After 2 minutes of  
homogenization, 200  $\mu$ L of chloroform was added and  
homogenization continued for a further 3 minutes.  
The homogenate was transferred to 250  $\mu$ L centrifuge  
10 bottles (Nalgene) and centrifuged for 15 minutes at  
2,500 x g. The upper aqueous phase was removed and  
mixed with 12  $\mu$ L of 5 M NaCl, equally divided into  
two centrifuge bottles, and 150  $\mu$ L of ethanol was  
added to each bottle. The mixture was stored at -  
15 20°C overnight. The RNA was collected by  
centrifugation at 4,000 x g for 15 minutes at 4°C.  
The RNA was dissolved in 50  $\mu$ L TE1 (50 mM tris-HCL  
[pH 8.0], 10 mM Na<sub>2</sub>EDTA) and clarified by  
centrifugation at 12,000 x g for 10 minutes at 4°C.  
20 The supernatant was transferred to, a new centrifuge  
bottle and 3  $\mu$ L of 5 M NaCl and 30  $\mu$ L of isopropanol  
were added. The contents were mixed and stored at -  
20°C overnight. The RNA was collected by  
centrifugation at 14,000 x g for 10 minutes. The  
25 RNA was washed with 20  $\mu$ L of 70% ice-cold ethanol  
and collected by centrifugation as before. After

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drying under vacuum for 10 minutes, the RNA was resuspended in 50  $\mu$ L of TE1 buffer and 10  $\mu$ L of 12 M LiCl was added. The solution was incubated at 4°C for 48 hours and the RNA was collected by

5 centrifugation at 14,000 x g for 10 minutes and resuspended in 30  $\mu$ L TE1 buffer. After the addition of 15  $\mu$ L of 5 M potassium acetate, the RNA was incubated overnight at 0°C, recovered by

centrifugation at 14,000 x g for 10 minutes and

10 suspended in 50  $\mu$ L TE1 buffer. Three  $\mu$ L of 5 M NaCl and 110  $\mu$ L of 95% ethanol were added and the RNA was incubated at -20°C overnight. The RNA was recovered by centrifugation at 14,000 x g for 10 minutes,

washed with 20  $\mu$ L of 70% ice-cold ethanol, recovered

15 by centrifugation as above, dried under vacuum for 10 minutes and resuspended in 600  $\mu$ L of TE1 buffer. The RNA was transferred into a microcentrifuge tube and centrifuged at 14,000 rpm for 30 minutes at 4°C after which 300  $\mu$ L was removed to each of two new

20 microcentrifuge tubes. The original centrifuged tube was rinsed with an additional 300  $\mu$ L of TE1 buffer. Eighteen  $\mu$ L of 5 M NaCl and 636  $\mu$ L of 100% ethanol were added to each of the three tubes.

After mixing by inverting, the tubes were stored

25 overnight at -20°C. The RNA was collected by centrifugation at 14,000 rpm for 30 minutes and



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washed with 1  $\mu$ L of 70% ice-cold ethanol. After centrifugation and drying as above, the RNA was resuspended in 400  $\mu$ L sterile H<sub>2</sub>O. A total of 1.04 mg total RNA was obtained.

5        Messenger RNA (polyA<sup>+</sup> RNA) was isolated using the PolyATtract<sup>®</sup> mRNA Isolation System IV (Promega Corporation, Madison, WI). A total of two isolations were done as follows. For each isolation, 0.48 mg total RNA was dissolved in 800  $\mu$ L  
10 of RNase-free water. After heating at 65°C for 10 minutes, 3  $\mu$ L of 50 pmole/mL biotinylated oligo(dT) and 20.7  $\mu$ L of 20 X SSC (1 X SSC contains 150 mM NaCl and 15 mM sodium citrate) were added and the mixture was allowed to slowly cool to room  
15 temperature over a period of approximately 30 minutes. An aliquot of streptavidin paramagnetic particles (provided in the PolyATtract<sup>®</sup> mRNA Isolation System IV) was washed 3 times in 0.5 X SSC and resuspended in 0.1 mL of 0.5 X SSC. The RNA  
20 solution containing the biotinylated oligo(dT) was added to the washed streptavidin paramagnetic particles. After a 10 minute incubation at room temperature, the paramagnetic particles containing the trapped mRNA were captured to the side of the  
25 tube using a magnet.

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The supernatant was removed and the particles were washed four times with 0.3 mL of 0.1 X SSC. The mRNA was removed from the biotinylated oligo(dT) particles by suspending in 200  $\mu$ L RNase-free water. 5 An additional elution was carried out by adding 150  $\mu$ L of water sequentially to each of the two tubes. The elution fractions (550  $\mu$ L) were pooled and centrifuged at 14,000 rpm in a microcentrifuge for 30 minutes at 4°C. The supernatant was divided into 10 two microcentrifuge tubes and, after the addition of 1/10th volume of 3 M NaCl and 600  $\mu$ L of ethanol, the mRNA was recovered by incubating the tubes at -20°C overnight, followed by centrifugation as above. The mRNA was washed once with 1 mL of ice-cold 70% 15 ethanol, dried and resuspended in 20  $\mu$ L sterile H<sub>2</sub>O. One  $\mu$ L was added to 1 mL of water and a spectrum was obtained from 230 nm through 330 nm in a Shimadzu UV 160U spectrophotometer. Approximately 6  $\mu$ g of mRNA was recovered from 1.04 mg of total RNA.

20 b) Construction of a cDNA Library

First and second strand cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). Six micrograms of mRNA in 20  $\mu$ L of water were incubated at 65°C for 5 minutes. Two 25 microliters of 100 mM methyl mercury were added and incubation was continued at room temperature for 10

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minutes. Four microliters of 700 mM  $\beta$ -mercaptoethanol were added and the incubation was continued for an additional 5 minutes. To the denatured mRNA, 5  $\mu$ L of 10 X first strand buffer

5 (provided in the kit), 5  $\mu$ L of 100 mM DTT, 3  $\mu$ L nucleotide mixture (10 mM each dATP, dGTP, dTTP and 5-methyl-dCTP), 2  $\mu$ L of 1.4  $\mu$ g/ $\mu$ L linker-primer:

5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT-3'

10 (SEQ. ID NO. 1)

1  $\mu$ L RNase block and 5  $\mu$ L of water were added. The reaction was incubated at room temperature for 10 minutes to anneal the primer to the mRNA and then 3

15  $\mu$ L of 20 U/ $\mu$ L M-MuLV reverse transcriptase were added. Five microliters of this reaction mixture were removed to a tube containing 0.5  $\mu$ L (0.625 pmoles) of 800 Ci/mmol [ $\alpha$ - $^{32}$ P]dATP. Both reactions were incubated at 37°C for 1 hour. The

20 radioactively labeled reaction was frozen at -20° C for later gel analysis. To the 45  $\mu$ L main reaction, 40  $\mu$ L of second strand buffer, 15  $\mu$ L of 100 mM DTT, 6  $\mu$ L of nucleotide mixture (10 mM dATP, dGTP, dTTP and 26 mM dCTP), 268.3  $\mu$ L water and 2  $\mu$ L (2.5

25 pmoles) of 800 Ci/mmol [ $\alpha$ - $^{32}$ P]dATP were added. After mixing, 4.5  $\mu$ L of 1 U/ $\mu$ L RNase H and 19.2  $\mu$ L of 5.2 U/ $\mu$ L *E. coli* DNA polymerase I were added and the reaction was incubated at 16° C for 2.5 hours. The

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reaction was extracted with 400  $\mu$ L of phenol:chloroform (1:1). The phases were separated by centrifugation in a microcentrifuge for 5 min and the aqueous phase removed and re-extracted with 5 chloroform. The aqueous phase was recovered by centrifugation as before.

The double-stranded cDNA was precipitated by the addition of 33.3  $\mu$ L of 3M sodium acetate (pH 5.2) and 867  $\mu$ L of 100% ethanol and incubation 10 overnight at -20°C. The cDNA was recovered by centrifugation at 14,000 X g in a microcentrifuge at 4°C for 60 minutes. The cDNA was washed with 1 mL of 80% ethanol, recovered by centrifugation at room temperature in a microcentrifuge at 14,000 X g, 15 dried under vacuum and dissolved in 45  $\mu$ L of water. Three microliters of the resuspended double-stranded cDNA was removed and stored at -20°C for later analysis by gel electrophoresis.

To the remaining 42 ML of the double-stranded 20 cDNA, 5  $\mu$ L of 10 X Klenow buffer (buffer #3; supplied by Stratagene), 2.5  $\mu$ L of 2.5 mM nucleotides (dCTP, dGTP, dATP and dTTP), and 0.5  $\mu$ L of 5 U/ $\mu$ L *E. coli* DNA polymerase I Klenow fragment were added. After 30 minutes at 37°C, 50  $\mu$ L of 25 water were added and the reaction was extracted with an equal volume of phenol:chloroform (1:1) and then

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chloroform as described above. After the addition of 7  $\mu$ L of 3M sodium acetate (pH 5.2) and 226  $\mu$ L of 100% ethanol, the blunt-ended double-stranded cDNA was incubated on ice for 30 minutes and recovered by centrifuging at 14,000 rpm at 4°C for 60 minutes in a microcentrifuge. The cDNA was washed with 300  $\mu$ L of 70% ethanol, centrifuged and dried as before. Seven microliters of 0.4  $\mu$ g/ $\mu$ L *Eco*RI linkers were added to the dried cDNA. The structure of the *Eco*RI linkers are:

5'-AATTCGGCACGAG-3' (SEQ. ID NO. 2)

3'-GCCGTGCTC-5'

After vortexing to resuspend the cDNA, 1  $\mu$ L of 10 X ligation buffer, 1  $\mu$ L 10 mM ATP and 1  $\mu$ L of 4 Weiss U/ $\mu$ L T4 DNA ligase were added and the reaction was incubated over night at 8°C. The ligase was inactivated by heating at 70°C for 30 minutes. The 5' ends of the *Eco*RI linkers, that are now attached to the cDNA, were phosphorylated using polynucleotide kinase. One microliter of 10 X buffer #3 of the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA), 2  $\mu$ L of 10 mM ATP, 6  $\mu$ L of water and 1  $\mu$ L of 10 U/ $\mu$ L T4 polynucleotide kinase were added to the ligation reaction. After 30 minutes at 37°C the kinase reaction was stopped by heating the reaction at 70°C for 30 minutes. *Xho*I "sticky ends"

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were generated at the end of the cDNA corresponding to the 3' end of the mRNA by digestion of the *Xho*I site in the linker-primer. Twenty-eight  $\mu$ L of *Xho*I buffer and 3  $\mu$ L of 40 U/ $\mu$ L *Xho*I were added to the cDNA and the reaction was incubated at 37°C for 1.5 hours.

The cDNA, with *Eco*RI sticky ends at the 5' end and *Xho*I sticky ends at the 3' end (relative to the original mRNA), was size fractionated by passage through a Sephacryl S-400 spin column prepared as follows. Five  $\mu$ L of 10 X STE [100 mM Tris (pH 7.0), 5 mM EDTA and 100 mM NaCl] were added to the cDNA and the cDNA was applied to the top of a 1 mL syringe containing Sephacryl S-400 (Pharmacia Biotech, Piscataway, NJ). A 500  $\mu$ L microcentrifuge tube was placed on the bottom of the syringe and the column was placed in a centrifuge tube and centrifuged at about 400 X g for 2 minutes. Sixty  $\mu$ L of 1 X STE were added to the top of the syringe, a new microcentrifuge tube was placed on the bottom of the column and the column was again centrifuged as before. This process was repeated until six fractions had been collected. About 10% of each fraction was electrophoresed on a 1% agarose gel to determine the size distribution of the cDNA in each fraction. The remainder of each fraction was

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extracted with an equal volume of phenol:chloroform and then chloroform as described above and precipitated by the addition of 2 volumes of 100% ethanol. After overnight incubation at -20°C the

5 cDNA was recovered by centrifugation in a microcentrifuge at 14,000 rpm for 60 minutes at 4°C. Each cDNA fraction was washed with 200 NL of 80% ethanol and dried as described above. cDNA fraction 1 was resuspended in 3  $\mu$ L of sterile water, and cDNA

10 fraction 2 was resuspended in 10.5  $\mu$ L of sterile water. One-half  $\mu$ L of each of the two fractions was used to determine the quantity of DNA using the ethidium bromide plate detection method. Fractions 1 and 2, containing the largest cDNA molecules, were

15 combined. The 12.5 mL combined fractions contained approximately 100 ng of cDNA. This fraction was reduced to 2.5  $\mu$ L in a Speed-Vac and stored on ice. cDNA fraction 3 was resuspended in 10.5  $\mu$ L of sterile water, and saved at -20°C for later use.

20 One-hundred ng of cDNA from fraction 1 and 2 were ligated into 1  $\mu$ g of Uni-ZAP™ (Stratagene, La Jolla, CA), a lambda ZAP vector that had been digested with *Eco*RI and *Xho*I. Fraction 1 and 2 cDNA (2.5  $\mu$ L) were added to 0.5  $\mu$ L of 10 X ligation

25 buffer, 0.5  $\mu$ L 10 mM ATP, 1  $\mu$ L of 1  $\mu$ g/ $\mu$ L Uni-Zap XR vector and 0.5  $\mu$ L of 4 Weiss U/ $\mu$ L T4 DNA ligase.

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The reaction was incubated at 8°C for about 44 hours. A 1 µL aliquot of the ligation reaction was added to one aliquot of the 'Freeze-Thaw' extract from the Gigapack II Gold bacteriophage λ packaging kit (Stratagene, La Jolla, CA). Fifteen microliters of Sonic extract were added and the contents were gently mixed. The packaging was carried out at room temperature. After 2 hours, 500 µL of SM buffer and 20 µL of chloroform were added to each packaging reaction and the debris was removed by a short centrifugation in a microcentrifuge. The packaged phages were moved to a new microcentrifuge tube. Ten µL of chloroform were added and the packages phages were stored at 4°C until used. A titer of this primary library indicated the presence of 0.7 X 10<sup>6</sup> recombinant plaques.

c) **Amplification of primary library.**

Six-hundred µL of *E. coli* XL1-Blue MRF' (Stratagene, La Jolla, CA), grown to a density of 0.5 at O.D.<sub>600</sub>, and 32.5 µL of primary library stock were added to each of 16 tubes. After incubation at 37°C for 15 min, 6.0 mL of 48°C top agar (5 g/L NaCl, 2 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g/L yeast extract, 10 g/L NZ amine [pH 7.5], and 0.7% agarose) were added to each tube and the contents were plated on 150 X 15 mm NZY plates (5 g/L NaCl, 2 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g/L



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yeast extract, 10 g/L NZ amine [pH 7.5], and 15 g/L Difco agar). The plates were incubated overnight at 37°C and then overlayed with 10 mL of SM buffer and incubated for a further 8 hours at 4°C with gentle shaking. The SM buffer was collected with a sterile pipette and stored in a sterile 250 mL centrifuge bottle. Each plate was rinsed with an additional 10 mL of SM buffer which were collected and added to the previous SM buffer. Chloroform, to a final concentration of 5%, was added and the phage solution was incubated at room temperature for 15 minutes and then centrifuged at 2,000 X g for 10 minutes to remove cell debris. The supernatant was recovered to a sterile polypropylene bottle and chloroform was added to a final concentration of 0.3%. The amplified library was stored at 4°C.

d) **Plating of amplified library for screening for specific genes.**

The amplified library was titered as described above. Approximately 50,000 recombinant plaques were added to 600 µL of *E. coli* XL1-Blue MRF' that were grown as described above. After 15 min at 37°C, 6.5 mL of 48°C top agar were added and the cells were plated on 150 X 15 mm NZY plates. Four plates containing a total of 200,000 recombinant plaques were prepared and incubated at 37°C overnight. The plates were then chilled for 4 hours

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at 4°C, then used for preparing plaque lifts as described below.

5 e) **Identification and Construction of  
Oligonucleotides Homologous to Coffee ACC  
Synthase Genes**

In previous studies, described in United States patent application serial number 08/485,107 the specification of which is incorporated herein by reference, we identified base sequences common to ACC synthase occurring in a variety of plants, referred to herein as consensus sequences. Based on these studies, we developed a set of three (3) fully degenerate primers for PCR amplification of regions of coffee first strand cDNA corresponding to consensus sequences. The sequence of the primers used is:

ACS167: 5'-GCCAAGCTTCRTGRTARTCYTGRAA-3'

20 (SEQ. ID NO. 3)

ACS289: 5'-TTYCARGAYTAYCAYGGHYT-3'

(SEQ. ID NO. 4)

ACS885: 5'-CCHGGDARNCCYAWRTCTTT-3'

(SEQ. ID NO. 5)

25 f) **Reverse Transcriptase reaction to obtain first-strand coffee cDNA.**

The reverse transcriptase reaction to obtain first-strand cDNA was performed in a final volume of 20 µL using the GeneAmp RNA PCR Core Kit (Perkin

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Elmer, Foster City, CA). First, 0.9  $\mu$ g of coffee fruit mRNA in 3  $\mu$ L water was mixed with 1  $\mu$ L of 50  $\mu$ M random hexamer and 6  $\mu$ L of sterile water in a microcentrifuge tube and incubated at 65°C for 5 minutes. The mixture was left at room temperature for 2 minutes and the liquid was recovered to the bottom of the tube by a brief centrifugation. To this mixture 2  $\mu$ L PCR buffer II (from the above mentioned kit), 4  $\mu$ L 25 mM MgCl<sub>2</sub>, 2  $\mu$ L 10 mM dNTP's, 1  $\mu$ L RNAsin (20 u/ $\mu$ L), and 1  $\mu$ L reverse transcriptase (50 u/ $\mu$ L) were added. The reaction was incubated at 42°C for 1 hour after which the reverse transcriptase was heat inactivated in a 95°C water bath for 5 minutes.

15 g) **Polymerase chain reaction to amplify coffee ACC-synthase gene.**

A polymerase chain reaction (PCR) (Saiki et al., 1988) was performed using the GeneAmp Kit described above in a 50  $\mu$ L reaction containing 10  $\mu$ L first-strand cDNA mix, 4  $\mu$ L PCR buffer II, 1  $\mu$ L 25 mM MgCl<sub>2</sub>, 2.5  $\mu$ L of 20  $\mu$ M AC5167 primer (SEQ. ID NO. 3), 2.5  $\mu$ L 20  $\mu$ M AC5885 primer (SEQ. ID. NO. 5), 29.5  $\mu$ L sterile H<sub>2</sub>O, and 0.5  $\mu$ L Tag DNA polymerase (5 u/ $\mu$ L). PCR conditions were 35 cycles of 94°C for 1 minute, 44°C for 1 minute, and 72°C for 2 minutes. The product of the PCR reaction was analyzed by agarose gel electrophoresis using 1.5% SeaPlaque

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agarose (FMC BioProducts, Rockland, ME) and *Hae* III-digested  $\phi$ X174 DNA (Promega Corporation, Madison, WI) as size markers. A single PCR product of approximately 650 bp was obtained.

5 h) **Amplification of PCR product with different primers.**

The 650 bp fragment obtained above was excised from the gel and placed in a 1.5 mL microcentrifuge tube. After the addition of 200  $\mu$ L of sterile water, the 650 bp fragment was heated to 90°C for 5 minutes, cooled to room temperature and centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge. The supernatant containing the amplified DNA was removed and placed in a new sterile 1.5 mL microcentrifuge tube. A 25  $\mu$ L PCR reaction was carried out using 0.4  $\mu$ L of the previously amplified DNA as template, 2.5  $\mu$ L 10 X PCR buffer (10 mM Tris-HCl pH 9.0, 0.1% triton X-100), 2  $\mu$ L 25 mM MgCl<sub>2</sub>, 5  $\mu$ L of 1 mM dNTPs, 1  $\mu$ L of 20  $\mu$ M ACS289 primer (SEQ. ID. NO. 5), 1  $\mu$ L of 20  $\mu$ M ACS885 primer (table 2), 12.8  $\mu$ L H<sub>2</sub>O, and 0.3  $\mu$ L Tag DNA polymerase (5 u/ $\mu$ L) (Promega Corporation, Madison, WI). The PCR was performed using 35 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes. Five  $\mu$ L of this reaction was electrophoresed in a 1.5% agarose gel as described above. A single product of approximately 603 bp was observed. Eighty  $\mu$ L of

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sterile water, 10  $\mu$ L of 3 M sodium acetate (pH 5.2), and 220  $\mu$ L of 100% ethanol was added to the remainder of the reaction. After incubation at 20°C overnight, the DNA was recovered by

5 centrifugation at 4°C for 30 minutes at 14,000 rpm. The DNA was washed with 400  $\mu$ L of ice-cold 75% ethanol and resuspended in 25  $\mu$ L of sterile water. The DNA concentration was determined to be 10 ng/ $\mu$ L using the ethidium bromide plate assay.

10 1) **Labeling Coffee Fruit-Specific ACC Synthase DNA**

A random primed probe was produced using the PCR-generated ACC synthase DNA and the Prime-a-Gene Kit (Promega Corporation, Madison, WI). Two and one-half  $\mu$ L of the DNA (25 ng) was added to 27.5  $\mu$ L  
15 of sterile water and the DNA was denatured by boiling for 5 min. Ten  $\mu$ L of 5 X labeling buffer, 2  $\mu$ L of unlabeled dNTP's [20  $\mu$ M each; dCTP, dGTP, dTTP], 2  $\mu$ L 1 mg/mL acetylated BSA, 1  $\mu$ L 5u/ $\mu$ L E. coli DNA polymerase I Klenow fragment and 5  $\mu$ L (50  
20  $\mu$ Ci) of [ $\alpha$ -<sup>32</sup>P]dATP (3,000 Ci/mmol) (Dupont-NEN) were added to give a final volume of 50  $\mu$ L. After 1 hr at room temperature, the reaction was terminated by the addition of 2  $\mu$ L of 0.5 M Na<sub>2</sub>EDTA and boiling for 2 min.

25 j) **Screening of amplified library with the ACC synthase-specific probe.**

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Plaque lifts of the four 150x15 mm NZY plates containing 50,000 recombinant clones each were prepared. Four 132 mm Magna nylon transfer membranes (Micron Separations, Incorporated, 5 Westborough, MA) were wetted by placing them on chromatography paper saturated with 5 X SSC buffer for approximately 10 sec. The membranes were placed on the plates containing the recombinant plaques for 5 min, removed and incubated, phage containing side 10 up, for 2 min on chromatography paper saturated with 0.5 M NaOH and 1.5 M NaCl. The membranes were then neutralized by transferring onto chromatography paper saturated with 0.5 M tris-HCl (pH 8.0) and 1.5 M NaCl, for 5 min. After a brief 20 sec treatment 15 on chromatography sheets saturated with 2 X SCC containing 0.2 M tris-hcl (pH 7.5), the filters were blotted dry. After 1 hour of air drying, DNA was cross-linked to the membranes by treatment with 12,000  $\mu$ Joules of a 260 nm UV light in a UV 20 Stratalinker 1800 (Stratagene, La Jolla, CA).

The four membranes were prehybridized at 65°C for 2 hours in 100 mL 6 X SSPE (52.2 g/L NaCl, 8.3 g/L  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 2.2 g/L  $\text{Na}_2\text{EDTA}$ , [pH 7.4]), 5 X Denhardt's solution (1 g/L Ficoll, 1 g/L 25 polyvinylpyrrolidone, 1 g/L BSA [pentax fraction V]), 0.5% SDS and 100  $\mu$ g/mL denatured herring sperm

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DNA in a Hybaid Mark II hybridization oven (National Labnet Company, Woodbridge, NJ) using HB-OV-BL bottles.

Hybridization was carried out at 65°C for 12  
5 hours in 10 mL of 6 X SSPE containing 0.5% SDS, 100  
µg/mL denatured herring sperm DNA, and 52 µL of the  
random primed probe described above. At the end of  
the hybridization period the hybridization solution  
was removed and the membranes were briefly washed  
10 with 100 mL of 2 X SSC containing 0.5% SDS at 65°C.  
They were then washed for an additional 30 min with  
the same amount of fresh buffer again at 65°C. The  
membranes were washed twice more for 30 min at 65°C  
with 100 mL of 0.2 X SSC containing 0.5% SDS,  
15 wrapped in a cellophane envelope and exposed to pre-  
flashed Fuji RX<sub>OCU</sub> X-ray film at -70°C for 24 hours.  
Ten positive clones were obtained. The region of  
the original plates corresponding to the identified  
plaques were removed and placed in 1 mL of SM buffer  
20 containing 20 µL chloroform. Of these ten, 5 were  
re-plated at lower densities and rescreened as above  
to obtain individual plaques.

k) **Characterization of Coffee-Fruit ACC synthase  
cDNA clones.**

25

The size of the putative coffee ACC synthase  
cDNA clones was determined by polymerase chain  
reaction using primers homologous to a portion of

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the T3 and T7 promoters present in the cloning vector and flanking the cDNA insertion site. The sequence of the primers are:

T3: 5'-TAATACGACTCACTATAGGG-3' (SEQ. ID NO. 6)

5 T7: 5'-AATTAACCCTCACTAAAGGG-3' (SEQ. ID NO. 7)

The conditions for PCR were as described above except that the temperature cycle was 95°C for 1 min., 50°C for 1 min. and 72°C for 2 min. Analysis was by agarose gel electrophoresis as before.

10 The three largest clones were recovered as phagemids by *in vivo* excision. Two hundred  $\mu$ L of phage stock from a single plaque was mixed with 200  $\mu$ L of *E. coli* XL1-Blue MRF' grown to a density at O.D.<sub>600</sub> of 1.0. One  $\mu$ L of ExAssist (Stratagene, La  
15 Jolla, CA) helper phage ( $>1 \times 10^6$  pfu/ $\mu$ L) was added and the tubes were incubated at 37°C for 15 min. Three mL of sterile LB broth were added and they were incubated for 3 hours at 37°C with shaking. After heating at 70°C for 20 min and centrifugation  
20 at 1,000 X g for 15 min, 1 mL of the supernatant, containing the excised pBluescript phagemid packaged as filamentous phage particles, was transferred to a sterile 1.5 mL microcentrifuge tube and stored at 4°C. Phagemids were recovered by adding 25  $\mu$ L of  
25 the stock solution to 200  $\mu$ L of *E. coli* Solar cells (Stratagene, La Jolla, CA) grown to a density of 1



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when measured at O.D.<sub>600</sub>. After incubation at 37°C for 15 min, 200 µL of the cell mixture was plated on 100 X 15 mm NZY agar plates containing 50 µg/mL ampicillin. The plates were incubated overnight at 37°C. Individual colonies were picked into 10 mL of LB broth containing 50 µg/mL ampicillin and grown overnight in a 37°C shaking incubator. The cells were concentrated in a 1.5 mL sterile microcentrifuge tube by repeated centrifugation and the phasmid DNA was purified using the plasmid mini kit from QIAGEN. The bacterial pellets were washed with water and resuspended in 0.3 mL of buffer P1. Next, 0.3 mL of alkaline lysis buffer P2 was added, mixed gently, and incubated for less than 5 min at room temperature. Following the addition of 0.3 mL of chilled buffer P3 and mixing by inverting the tubes 6 times, the extracts were incubated on ice for 10 min and centrifuged at 14,000 rpm for 15 min in a microcentrifuge. The supernatants were removed and applied to QIAGEN-tip 20 columns that had been previously equilibrated with 1 mL of QDT buffer. The extracts were allowed to enter the resin of the columns by gravity flow. Once the flow had stopped, the columns were washed 4 times with 1 mL buffer QC. The DNAs were eluted by washing the QIAGEN-tip 20 columns with 0.8 mL buffer QF which was collected

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into 1.5 mL microcentrifuge tubes. The DNA was precipitated by the addition of 0.7 volumes (560  $\mu$ L) of isopropanol. The tubes were immediately centrifuged at 14,000 rpm for 30 min and the supernatant carefully removed. The pellets, containing the DNA, were washed 20 with 1 mL of ice-cold 70% ethanol, centrifuged as above, and air dried for 5 min. The DNA was resuspended in 50  $\mu$ L sterile H<sub>2</sub>O. The concentration of DNA from one plasmid isolation was 0.1  $\mu$ g/ $\mu$ L by fluorometric analysis.

Sequencing reactions were performed by mixing 8  $\mu$ L of phagmid DNA (0.8  $\mu$ g) with 4  $\mu$ L of either T3 or T7 sequencing primers (0.8 pmol/ $\mu$ L). Automated DNA sequencing was carried out on these samples at the University of Hawaii Biotechnology Service Center. About 350 bp of sequence from both the 5' and the 3' end of the cDNA was obtained. New sequencing primers were synthesized based on sequences near the end of the previous sequences and used in the same manner to complete the sequence of both strands of the cDNA. The complete sequence of the coffee fruit-expressed ACC synthase cDNA is given in Figure 1. The deduced amino acid sequence of the coffee fruit-expressed ACC synthase is given in Figure 2.

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The sequence of the coffee ACC synthase cDNA clone and deduced protein was compared with other ACC synthase genes present in GenBank. The cDNA isolated from coffee fruit shows from 68.3% to 58.1% identity to other ACC synthases present in GenBank. And, the protein sequence deduced from this cDNA shows from 67.9% to 50.5% identity to other ACC synthases. However, this cDNA is unique in that no other sequence greater than 1500 bp showed greater than 68.3% identity to it.

**EXAMPLE 2***Isolation of Coffee Fruit-Specific ACC Oxidase*

- 15 a) **Synthesis of ACC Oxidase specific oligonucleotide primers.**

The isolation of total RNA, mRNA, and the synthesis of coffee fruit-specific cDNA was as described above.

Twelve ACC oxidase sequences, obtained from GenBank, were aligned using the Pileup program of GCG (Genetics Computer Group, Madison, WI). A region approximately 1000 bp from the translation start codon was found to be conserved and a degenerate oligonucleotide primer

5'-TCATIGCKKCRAKIGGTTC-3' (SEQ. ID NO. 8) corresponding to this region was synthesized. Inosine (I) was placed at positions showing no sequence conservation, since position could be any

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of A, T, G or C. Positions showing two-fold ambiguity were prepared with mixed residues (T/G or A/G). We also prepared a second primer homologous to a region of the papaya fruit-expressed ACC oxidase cDNA that had been previously cloned in our laboratory and situated approximately 372 bp from the translational start codon:

5'-GACACTGTGGAGAGGCTGAC-3' (SEQ. ID NO. 9)

The two primers were used in a PCR reaction to amplify a portion of the coffee fruit-expressed ACC oxidase. The PCR contained 0.2  $\mu$ L (10 ng) cDNA fraction 3 (described in Example 1), 5  $\mu$ L 10 X PCR buffer, 3  $\mu$ L 25 mM  $MgCl_2$ , 1  $\mu$ L of each of the four 10 mM dNTPs, 1  $\mu$ L of a 20  $\mu$ M solution of each primer, 0.3  $\mu$ L Taq DNA polymerase (promega Corporation, Madison, WI) and 38.5  $\mu$ L water. PCR conditions were 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A 5 min incubation at 72°C was carried out after the last cycle. A 20  $\mu$ L aliquot of the product was electrophoresed in a 1.5% agarose gel as described previously and revealed an approximately 800 bp product. The DNA was excised from the gel and mixed with 200  $\mu$ L of sterile water in a 1.5 mL microcentrifuge tube. After boiling for 5 min, 2  $\mu$ L was used as a template in a 50  $\mu$ L PCR reaction as above using the same primers. Gel

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electrophoresis performed as described above using 20  $\mu$ L of the PCR reaction indicated the presence of a single 800 bp product. To the remaining 30  $\mu$ L of the PCR reaction 20  $\mu$ L chloroform and 100  $\mu$ L water was added. The contents were mixed and centrifuged for 2 minutes at 14,000 rpm in a microcentrifuge. The upper aqueous phase containing the DNA was removed to a clean microcentrifuge tube. A portion of this DNA was radioactively labeled by random primed synthesis as described above.

**b) Screening of amplified library with random primed probe.**

The amplified coffee-fruit cDNA described in Example 1 was used to prepare four 150 X 10 mm NZY plates as previously described. Prehybridization, hybridization and recovery of clones was as previously described except that the ACC oxidase sequence obtained by PCR was used as the probe.

**20 c) Characterization of Coffee-Fruit ACC-oxidase cDNA clones.**

The size of the coffee ACC-oxidase cDNA clones was determined by polymerase chain reaction using primers homologous to the T3 and T7 promoters as described in Example 1.

The sequence of the largest coffee ACC oxidase cDNA clone was obtained as described in Example 1 and compared with ACC oxidase genes present in

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GenBank. Figure 3 gives the sequence of the coffee fruit-specific ACC oxidase. Figure 4 gives the deduced amino acid sequence of this protein. The cDNA was determined to encode ACC oxidase because it is from 50.4% to 82.5% identical to other ACC synthases nucleic acid sequences present in GenBank. Also, the deduced protein sequence is from 32.5% to 86.5% identical to other ACC oxidases.

The foregoing examples are for illustrative purposes only, and should not be viewed as limiting the scope of applicants' invention, which is set forth in the claims appended hereto.

## SEQUENCE LISTING 37

## (1) GENERAL INFORMATION:

- (i) APPLICANT: STILES, JOHN I.  
MOISYADI, ISTEFO  
NEUPANE, KABI R.
- (ii) TITLE OF INVENTION: PURIFIED PROTEINS, RECOMBINANT  
DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE  
RIPENING OF COFFEE
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: JONES, DAY, REAVIS & POGUE
  - (B) STREET: NORTH POINT, 901 LAKESIDE AVENUE
  - (C) CITY: CLEVELAND
  - (D) STATE: OHIO
  - (E) COUNTRY: USA
  - (F) ZIP: 44114
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb  
storage
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: MS-DOS v. 5.1
  - (D) SOFTWARE: WordPerfect v. 6.1
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/695,412
  - (B) FILING DATE: 12-AUG-1996
  - (C) CLASSIFICATION: 435
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US08/485,107
  - (B) FILING DATE: 07-JUN-1995
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: GRIFFITH, CALVIN P.
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  - (C) REFERENCE/DOCKET NUMBER: 265036600002
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (216) 586-7050
  - (B) TELEFAX: (216) 579-0212

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 amino acid residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: N/A
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Fragment A

(B) LOCATION: 17..1480

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile	Asn	Tyr	Ala	Ser	Gly	Ala	Ser	Gly	Ile	Leu	Asp	Gln	Xaa	Gly
1				5					10					15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile	Asn	Tyr	Ala	Ser	Gly	Ala	Ser	Gly	Ile	Leu	Asp	Gln	Thr
1				5					10				14

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID

(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE:

(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATNAAYTAYG CNAGYGGNGC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear



- (ii) MOLECULE TYPE: OTHER NUCLEAR ACID
  - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
  - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:  
ATNAAYTAYG CNAGYGGNGC 20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: OTHER NUCLEIC ACID
  - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
  - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
CGNCCAGNCG NYTAYTTNAT 20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
  - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
  - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:  
CGNCCYCTYG CYTAYTTNAT 20

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 14 amino acid residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
  - (D) OTHER INFORMATION: Xaa is either Thr or Asp
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
Gln Tyr Val Pro Cys Tyr Phe Xaa Phe Ile Asp Asp Gln Asp  
1 5 10 14

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: OTHER NUCLEIC ACID
  - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: Internal
- (ix) FEATURE
  - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:  
CAWTATGTNC CNTGTTATTT 20

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: OTHER NUCLEIC ACID
  - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: Internal

(ix) FEATURE  
 (A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAWTAWCAHG GNACWTATTG 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 488 amino acid residues  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 178..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met	Glu	Phe	Ser	Leu	Lys	Asn	Glu	Gln	Gln	Gln	Leu	Leu	Ser	Lys	1	5	10	15
Met	Ala	Thr	Asn	Asp	Gly	His	Gly	Glu	Asn	Ser	Pro	Tyr	Phe	Asp	20	25	30	
Gly	Trp	Lys	Ala	Tyr	Asp	Ser	Asp	Pro	Tyr	His	Pro	Thr	Arg	Asn	35	40	45	
Pro	Asn	Gly	Val	Ile	Gln	Met	Gly	Leu	Ala	Glu	Asn	Gln	Leu	Cys	50	55	60	
Phe	Asp	Leu	Ile	Glu	Glu	Trp	Val	Leu	Asn	Asn	Pro	Glu	Ala	Ser	65	70	75	
Ile	Cys	Thr	Ala	Glu	Gly	Ala	Asn	Lys	Phe	Met	Glu	Val	Ala	Ile	80	85	90	
Tyr	Gln	Asp	Tyr	His	Gly	Leu	Pro	Glu	Phe	Arg	Asn	Ala	Val	Ala	95	100	105	
Arg	Phe	Met	Glu	Lys	Val	Arg	Gly	Asp	Arg	Val	Lys	Phe	Asp	Pro	110	115	120	
Asn	Arg	Ile	Val	Met	Ser	Gly	Gly	Ala	Thr	Gly	Ala	His	Glu	Thr	125	130	135	

Leu Ala Phe Cys	Leu Ala Asp Pro Glu Asp	Ala Phe Leu Val	Pro
140	145		150
Thr Pro Tyr Tyr	Pro Gly Phe Asp Arg Asp	Leu Arg Trp Arg	Thr
155	160		165
Gly Met Gln Leu	Leu Pro Ile Val Cys Arg	Ser Ser Asn Asp	Phe
170	175		180
Lys Val Thr Lys	Glu Ser Met Glu Ala Ala	Tyr Gln Lys Ala	Gln
185	190		195
Glu Ala Asn Ile	Arg Val Lys Gly Phe Leu	Leu Asn Asn Pro	Ser
200	205		210
Asn Pro Leu Gly	Thr Val Leu Asp Arg Glu	Thr Leu Ile Asp	Ile
215	220		225
Val Thr Phe Ile	Asn Asp Lys Asn Ile His	Leu Ile Cys Asp	Glu
230	235		240
Ile Tyr Ser Ala	Thr Val Phe Ser Gln Pro	Glu Phe Ile Ser	Ile
245	250		255
Ser Glu Ile Ile	Glu His Asp Val Gln Cys	Asn Arg Asp Leu	Ile
260	265		270
His Leu Val Tyr	Ser Leu Ser Lys Asp Leu	Gly Phe Pro Gly	Phe
275	280		285
Arg Val Gly Ile	Leu Tyr Ser Tyr Asn Asp	Ala Val Val Ser	Cys
290	295		300
Ala Arg Lys Met	Ser Ser Phe Gly Leu Val	Ser Thr Gln Thr	Gln
305	310		315
His Leu Ile Ala	Ser Met Leu Ser Asp Glu	Ala Phe Met Asp	Lys
320	325		330
Ile Ile Ser Thr	Ser Ser Glu Arg Leu Ala	Ala Arg His Gly	Leu
335	340		345
Phe Thr Arg Gly	Leu Ala Gln Val Gly Ile	Gly Thr Leu Lys	Ser
350	355		360
Ser Ala Gly Leu	Tyr Phe Trp Met Asp Leu	Arg Arg Leu Leu	Arg
365	370		375
Glu Ser Thr Phe	Glu Ala Glu Met Glu Leu	Trp Arg Ile Ile	Ile
380	385		390
His Glu Val Lys	Leu Asn Val Ser Pro Gly	Leu Ser Phe His	Cys
395	400		405

Ser	Glu	Pro	Gly	Trp	Phe	Arg	Val	Cys	Phe	Ala	Asn	Met	Asp	Asp	410	415	420
Glu	Ser	Val	Arg	Val	Ala	Leu	Arg	Arg	Ile	His	Lys	Phe	Val	Leu	425	430	435
Val	Gln	Gly	Lys	Ala	Thr	Glu	Pro	Thr	Thr	Pro	Lys	Ser	Arg	Cys	440	445	450
Gly	Ser	Ser	Lys	Leu	Gln	Leu	Ser	Leu	Ser	Phe	Arg	Arg	Leu	Asp	455	460	465
Glu	Arg	Val	Met	Gly	Ser	His	Met	Met	Ser	Pro	His	Ser	Pro	Met	470	475	480
Ala	Ser	Pro	Leu	Val	Arg	Ala	Thr								485		

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2040 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) Feature:

(A) NAME/KEY: CDS

(B) LOCATION: 178..1653

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAATCTCTT	CTAAAATCAA	CCATTCTCTT	CATTCTTCAC	TTGACAAGGC	50
CACTGCATTC	TTCATTCTTT	CTTGATATAT	AGCCATTTTT	TTCATTCTTT	100
CTTGATATAT	AGCCATTTTT	TTCATTCTTT	CTTCATTCAT	TGTCTGGAGA	150
AGTTGGTTGA	GTTTTCTTGA	AAATTCAAGC	AAAACA	ATG GAG TTC AGT	198
				Met Glu Phe Ser	
				1	
TTG AAA AAC GAA CAA CAA CAA CTC TTG TCG AAG ATG GCA ACC	240				
Leu Lys Asn Glu Gln Gln Gln Leu Leu Ser Lys Met Ala Thr					
5 10 15					

AAC GAT GGA CAT GGC GAA AAC TCG CCT TAT TTT GAT GGT TGG Asn Asp Gly His Gly Glu Asn Ser Pro Tyr Phe Asp Gly Trp 20 25 30	282
AAG GCA TAT GAT AGT GAT CCT TAC CAT CCC ACC AGA AAT CCT Lys Ala Tyr Asp Ser Asp Pro Tyr His Pro Thr Arg Asn Pro 35 40 45	324
AAT GGT GTT ATA CAG ATG GGA CTC GCA GAA AAT CAG TTA TGC Asn Gly Val Ile Gln Met Gly Leu Ala Glu Asn Gln Leu Cys 50 55 60	366
TTT GAT TTG ATC GAG GAA TGG GTT CTG AAC AAT CCA GAG GCT Phe Asp Leu Ile Glu Glu Trp Val Leu Asn Asn Pro Glu Ala 65 70	408
TCC ATT TGC ACA GCA GAA GGA GCG AAC AAA TTC ATG GAA GTT Ser Ile Cys Thr Ala Glu Gly Ala Asn Lys Phe Met Glu Val 75 80 85	450
GCT ATC TAT CAA GAT TAT CAT GGC TTG CCA GAG TTC AGA AAT Ala Ile Tyr Gln Asp Tyr His Gly Leu Pro Glu Phe Arg Asn 90 95 100	492
GCT GTA GCA AGG TTC ATG GAG AAG GTG AGA GGT GAC AGA GTC Ala Val Ala Arg Phe Met Glu Lys Val Arg Gly Asp Arg Val 105 110 115	534
AAG TTC GAT CCC AAC CGC ATT GTG ATG AGT GGT GGG GCA ACC Lys Phe Asp Pro Asn Arg Ile Val Met Ser Gly Gly Ala Thr 120 125 130	576
GGA GCT CAT GAA ACT CTG GCC TTC TGT TTA GCT GAC CCT GAA Gly Ala His Glu Thr Leu Ala Phe Cys Leu Ala Asp Pro Glu 135 140	618
GAT GCG TTT TTG GTA CCC ACA CCA TAT TAT CCA GGA TTT GAT Asp Ala Phe Leu Val Pro Thr Pro Tyr Tyr Pro Gly Phe Asp 145 150 155	660
CGG GAT TTG AGG TGG CGA ACA GGG ATG CAA CTT CTT CCA ATT Arg Asp Leu Arg Trp Arg Thr Gly Met Gln Leu Leu Pro Ile 160 165 170	702

GTT TGT CGC AGC TCC AAT GAT TTT AAG GTC ACT AAA GAA TCC	744
Val Cys Arg Ser Ser Asn Asp Phe Lys Val Thr Lys Glu Ser	
175 180 185	
ATG GAA GCT GCT TAT CAG AAA GCT CAA GAA GCC AAC ATC AGA	786
Met Glu Ala Ala Tyr Gln Lys Ala Gln Glu Ala Asn Ile Arg	
190 195 200	
GTA AAG GGG TTC CTC TTA AAT AAT CCA TCA AAT CCA TTG GGA	828
Val Lys Gly Phe Leu Leu Asn Asn Pro Ser Asn Pro Leu Gly	
205 210	
ACT GTT CTT GAC AGG GAA ACT TTG ATT GAT ATA GTC ACA TTC	870
Thr Val Leu Asp Arg Glu Thr Leu Ile Asp Ile Val Thr Phe	
215 220 225	
ATC AAT GAC AAA AAT ATC CAC TTG ATT TGT GAT GAG ATA TAT	912
Ile Asn Asp Lys Asn Ile His Leu Ile Cys Asp Glu Ile Tyr	
230 235 240	
TCT GCC ACC GTC TTC AGC CAG CCC GAA TTC ATC AGC ATC TCT	954
Ser Ala Thr Val Phe Ser Gln Pro Glu Phe Ile Ser Ile Ser	
245 250 255	
GAA ATA ATT GAG CAT GAT GTT CAA TGC AAC CGT GAT CTC ATA	996
Glu Ile Ile Glu His Asp Val Gln Cys Asn Arg Asp Leu Ile	
260 265 270	
CAT CTT GTG TAT AGC CTG TCC AAG GAC TTG GGC TTC CCT GGA	1038
His Leu Val Tyr Ser Leu Ser Lys Asp Leu Gly Phe Pro Gly	
275 280	
TTC AGA GTT GGC ATT TTG TAT TCA TAT AAT GAC GCT GTT GTC	1080
Phe Arg Val Gly Ile Leu Tyr Ser Tyr Asn Asp Ala Val Val	
285 290 295	
AGC TGT GCT AGA AAA ATG TCG AGT TTC GGC CTT GTT TCA ACA	1122
Ser Cys Ala Arg Lys Met Ser Ser Phe Gly Leu Val Ser Thr	
300 305 310	
CAA ACT CAG CAT CTG ATT GCA TCA ATG TTA TCG GAC GAA GCA	1164
Gln Thr Gln His Leu Ile Ala Ser Met Leu Ser Asp Glu Ala	
315 320 325	

TTT ATG GAC AAA ATC ATT TCC ACG AGC TCA GAG AGA TTA GCT	1206
Phe Met Asp Lys Ile Ile Ser Thr Ser Ser Glu Arg Leu Ala	
330 335 340	
GCA AGG CAT GGT CTT TTC ACA AGA GGA CTT GCT CAA GTA GGC	1248
Ala Arg His Gly Leu Phe Thr Arg Gly Leu Ala Gln Val Gly	
345 350	
ATT GGC ACC TTA AAA AGC AGT GCG GGC CTT TAT TTC TGG ATG	1290
Ile Gly Thr Leu Lys Ser Ser Ala Gly Leu Tyr Phe Trp Met	
355 360 365	
GAC TTA AGG AGA CTC CTC AGG GAG TCC ACA TTT GAG GCA GAA	1332
Asp Leu Arg Arg Leu Leu Arg Glu Ser Thr Phe Glu Ala Glu	
370 375 380	
ATG GAA CTT TGG AGG ATC ATA ATA CAT GAA GTC AAG CTC AAT	1374
Met Glu Leu Trp Arg Ile Ile Ile His Glu Val Lys Leu Asn	
385 390 395	
GTT TCA CCA GGC TTA TCT TTC CAT TGC TCA GAA CCA GGA TGG	1416
Val Ser Pro Gly Leu Ser Phe His Cys Ser Glu Pro Gly Trp	
400 405 410	
TTC AGA GTT TGC TTT GCC AAC ATG GAC GAC GAA AGT GTG AGA	1458
Phe Arg Val Cys Phe Ala Asn Met Asp Asp Glu Ser Val Arg	
415 420	
GTT GCT CTC AGA AGA ATC CAC AAA TTT GTG CTT GTT CAG GGC	1500
Val Ala Leu Arg Arg Ile His Lys Phe Val Leu Val Gln Gly	
425 430 435	
AAG GCA ACA GAG CCA ACA ACT CCA AAG AGT CGC TGC GGA AGC	1542
Lys Ala Thr Glu Pro Thr Thr Pro Lys Ser Arg Cys Gly Ser	
440 445 450	
AGC AAA CTT CAA CTC AGC TTA TCT TTC CGC AGA TTG GAC GAA	1584
Ser Lys Leu Gln Leu Ser Leu Ser Phe Arg Arg Leu Asp Glu	
455 460 465	
AGG GTG ATG GGA TCG CAT ATG ATG TCC CCT CAC TCC CCG ATG	1626
Arg Val Met Gly Ser His Met Met Ser Pro His Ser Pro Met	
470 475 480	



GCT TCA CCT TTG GTT CGG GCT ACA TAAATCATTT CTTGATCAGA 1670  
 Ala Ser Pro Leu Val Arg Ala Thr  
 485

TCATATAGCA AAGATTCCTG AGTAAATACT CGAAACCCTT TCTGGATAAC 1720  
 TGAAAAGAGA GTTGTTGATT CTTTGCTGTA TCATACAAAC ACGTTACAGG 1770  
 CATTTTTTGG CCATCTGATG CGTGCAAATT GCATCAAATG CTTTTATTAT 1820  
 TGTCAATTC ATTTGTGTAC CTTGGTTTTTC CTTGCCCTTC AGTCCTCCTT 1870  
 GTTTTTTGTT TCTTTGTTAT TATTTTCTTC CAGTTGATCA GTTAAACGAA 1920  
 GGAAGCTCAA TTGTTTCAAG CTATTAGTAA CAGATCATTT TGTAATAGCA 1970  
 ATAGTTTCAG GATTCTGAAA TGAAAGTTTA TCATTTTCC ATCATTTTAA 2020  
 AAAAAAAAAA AAAAAAAAAA 2040

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 318 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 46..1003

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

Met Ala Thr Phe Pro Leu Ile Asp Met Glu Lys Leu Asp Gly Glu  
 1 5 10 15  
 Glu Arg Ala Ala Thr Met Gly Val Ile Lys Asp Ala Cys Glu Ser  
 20 25 30  
 Trp Gly Phe Phe Glu Val Leu Asn His Gly Ile Ser Asn Glu Leu  
 35 40 45  
 Met Asp Thr Val Glu Arg Leu Thr Lys Glu His Tyr Lys Lys Cys  
 50 55 60

Met	Glu	Leu	Lys	Phe	Lys	Glu	Met	Val	Glu	Ser	Lys	Glu	Leu	Glu		65	70	75
Ala	Val	Gln	Thr	Glu	Ile	Asn	Asp	Leu	Asp	Trp	Glu	Ser	Thr	Phe		80	85	90
Phe	Leu	Arg	His	Leu	Pro	Val	Ser	Asn	Ile	Ser	Glu	Val	Pro	Asp		95	100	105
Leu	Asp	Asp	Glu	Tyr	Arg	Lys	Val	Met	Lys	Glu	Phe	Ala	Leu	Gln		110	115	120
Leu	Glu	Lys	Leu	Ala	Glu	Leu	Leu	Leu	Asp	Leu	Leu	Cys	Glu	Asn		125	130	135
Leu	Gly	Leu	Glu	Lys	Gly	Tyr	Leu	Lys	Lys	Ala	Phe	Tyr	Gly	Thr		140	145	150
Lys	Gly	Pro	Thr	Phe	Gly	Thr	Lys	Val	Ser	Asn	Tyr	Pro	Pro	Cys		155	160	165
Pro	Arg	Pro	Glu	Leu	Ile	Lys	Gly	Leu	Arg	Ala	His	Thr	Asp	Ala		170	175	180
Gly	Gly	Ile	Ile	Leu	Leu	Phe	Gln	Asp	Asp	Lys	Val	Ser	Gly	Leu		185	190	195
Gln	Leu	Leu	Lys	Asp	Gly	Glu	Trp	Val	Asp	Val	Pro	Pro	Met	Arg		200	205	210
His	Ser	Ile	Val	Ile	Asn	Ile	Gly	Asp	Gln	Leu	Glu	Val	Ile	Thr		215	220	225
Asn	Gly	Lys	Tyr	Lys	Ser	Val	Met	His	Arg	Val	Ile	Ala	Gln	Pro		230	235	240
Asp	Gly	Asn	Arg	Met	Ser	Leu	Ala	Ser	Phe	Tyr	Asn	Pro	Gly	Ser		245	250	255
Asp	Ala	Val	Ile	Tyr	Pro	Ala	Pro	Ala	Leu	Val	Glu	Lys	Glu	Ala		260	265	270
Glu	Asp	Lys	Gln	Ile	Tyr	Pro	Lys	Phe	Val	Phe	Glu	Asp	Tyr	Met		275	280	285
Lys	Leu	Tyr	Ala	Gly	Leu	Lys	Phe	Gln	Ala	Lys	Glu	Pro	Arg	Phe		290	295	300
Glu	Ala	Met	Lys	Ala	Val	Glu	Ser	Thr	Val	Asn	Leu	Gly	Pro	Ile		305	310	315
Ala	Thr	Val														318		

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1320 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix )Feature:

(A)NAME/KEY:CDS

(B)LOCATION:46..1003

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTAAACGAA GCATAAGCAC AAGCAAACAC AAACCTAGAAA GAGAG ATG	48
Met	
1	
GCT ACA TTC CCC CTA ATC GAC ATG GAG AAG CTT GAC GGT GAA	90
Ala Thr Phe Pro Leu Ile Asp Met Glu Lys Leu Asp Gly Glu	
5 10 15	
GAG AGG GCT GCC ACT ATG GGA GTC ATA AAA GAT GCT TGT GAA	132
Glu Arg Ala Ala Thr Met Gly Val Ile Lys Asp Ala Cys Glu	
20 25	
AGC TGG GGC TTC TTT GAG GTG TTG AAT CAT GGG ATA TCT AAT	174
Ser Trp Gly Phe Phe Glu Val Leu Asn His Gly Ile Ser Asn	
30 35 40	
GAG CTC ATG GAC ACA GTG GAG AGG CTA ACA AAG GAG CAT TAC	216
Glu Leu Met Asp Thr Val Glu Arg Leu Thr Lys Glu His Tyr	
45 50 55	
AAG AAA TGT ATG GAA CTA AAG TTC AAG GAA ATG GTG GAG AGC	258
Lys Lys Cys Met Glu Leu Lys Phe Lys Glu Met Val Glu Ser	
60 65 70	
AAG GAA TTG GAA GCT GTT CAG ACT GAG ATC AAT GAT TTG GAC	300
Lys Glu Leu Glu Ala Val Gln Thr Glu Ile Asn Asp Leu Asp	
75 80 85	

## CLAIMS

1. A substantially pure ACC synthase from *Coffea arabica* consisting essentially of the amino acid sequence: (SEQ. ID. NO. 10)
- 5 2. Substantially pure nucleic acid sequence that codes on expression for the ACC synthase produced by *Coffea arabica* comprising: (SEQ. ID. NO. 11)
- 10 3. The substantially pure nucleic acid sequence that codes on expression for the ACC synthase produced by *Coffea arabica* of claim 2, wherein the nucleic acid sequence is limited to the coding regions of SEQ. ID. No. 11.
- 15 4. A method for controlling the ripening of *Coffea arabica* comprising:
  - a) transforming coffee plants with a DNA sequence that is antisense to the DNA sequence SEQ. ID. NO. 11;
  - b) growing plants transformed with the
  - 20 DNA sequence of a) above; and
  - c) applying exogenous ethylene to the transformed plants after coffee fruit has matured.
- 25 5. The method for controlling the ripening of *Coffea arabica* of claim 4, wherein the DNA sequence used for transforming is limited to a sequence that is antisense to the coding region of SEQ. ID. No. 11.
- 30 6. The method for controlling fruit ripening of claims 4 or 5, wherein gaseous ethylene is applied to the entire plant, to cause ripening of substantially all of the fruit simultaneously.
- 35 7. A substantially pure ACC oxidase from *Coffea arabica* consisting essentially of the amino acid sequence: (SEQ. ID. NO. 12).

8. Substantially pure nucleic acid sequence that codes an expression for *Coffee arabica* ACC oxidase comprising: (SEQ. ID. No. 13).

9. The substantially pure nucleic acid  
5 sequence that codes on expression for ACC oxidase produced by *Coffee arabica* of claim 8, wherein the nucleic acid sequence is limited to the coding regions of SEQ. ID. No. 13.

10. A method for controlling ripening of  
10 *Coffea arabica* comprising:  
a) transforming coffee plants with a DNA sequence that is antisense to the DNA sequence: (SEQ. ID. No. 13);  
b) growing plants transformed with the  
15 DNA sequence of a) above; and  
c) applying exogenous ethylene to the transformed plants after coffee fruit has matured.

11. The method for controlling ripening of  
20 *Coffea arabica* of claim 10, wherein the DNA sequence used for transforming is limited to a sequence that is antisense to the coding region of SEQ. ID. NO. 13.

12. The method for controlling fruit ripening  
25 of claims 9 and 10, wherein the gaseous ethylene is applied to the entire plant, to cause ripening of substantially all of the fruit simultaneously.

13. A coffee plant having suppressed expression of ACC synthase.

30 14. A coffee plant having suppressed expression of ACC oxidase.

15. A coffee plant having suppressed expression of ACC synthase and suppressed expression of ACC oxidase.

16. A coffee plant comprising a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase.

5 17. A coffee fruit from the coffee plant of claim 16.

18. A coffee bean from the coffee plant of claim 16.

10 19. A coffee plant comprising a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

20. A coffee fruit from the coffee plant of claim 19.

15 21. A coffee bean from the coffee plant of claim 19.

22. A coffee plant comprising (i) a first DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, and (ii) a second DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

25 23. A coffee fruit from the coffee plant of claim 22.

24. A coffee bean from the coffee plant of claim 22.

30 25. A coffee plant comprising a DNA sequence that is antisense to all or part of the DNA sequence specified in SEQ ID NO:11.

26. A coffee plant comprising a DNA sequence that is antisense to all or part of the DNA sequence specified in SEQ ID NO:13.

35 27. A coffee plant comprising (i) a first DNA sequence that is antisense to all or part of the DNA

sequence specified in SEQ ID NO:11, and (ii) a second DNA sequence that is antisense to all or part of the DNA sequence specified in SEQ ID NO:13.

28. A coffee plant produced by the process of  
5 inserting into the plant genome a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase.

29. A coffee plant produced by the process of  
10 inserting into the plant genome a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

30. A coffee plant produced by the process of  
15 inserting into the plant genome (i) a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, and (ii) a DNA sequence that codes on transcription for a mRNA that is antisense to the  
20 mRNA that codes on expression for ACC oxidase.

31. A method for transforming a coffee plant with a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, comprising the steps  
25 of:

providing a transforming vector comprising a DNA sequence that codes on expression for ACC synthase, wherein the DNA sequence is inserted into the transforming vector in an inverted  
30 orientation; and

inserting the transforming vector into the tissue of the coffee plant, wherein the inverted DNA sequence thereafter becomes inserted into the genome of the coffee plant.

32. A method for transforming a coffee plant with a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase, comprising the steps of:

5 providing a transforming vector comprising a DNA sequence that codes on expression for ACC oxidase, wherein the DNA sequence is inserted into the transforming vector in an inverted orientation;

10 inserting the transforming vector into the tissue of the coffee plant, wherein the inverted DNA sequence thereafter becomes inserted into the genome of the coffee plant.

33. A method for transforming a coffee plant  
15 with (i) a first DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, and (ii) a second DNA sequence that codes on transcription for a mRNA that is antisense to the  
20 mRNA that codes on expression for ACC oxidase, comprising the steps of:

providing a first transforming vector comprising a first DNA sequence that codes on expression for ACC synthase, wherein the first  
25 DNA sequence is inserted into the first transforming vector in an inverted orientation;

providing a second transforming vector comprising a second DNA sequence that codes on expression for ACC oxidase, wherein the second  
30 DNA sequence is inserted into the second transforming vector in an inverted orientation;

inserting the first transforming vector into the tissue of the coffee plant, wherein the first inverted DNA sequence thereafter



becomes inserted into the genome of the coffee plant; and

5            inserting the second transforming vector into the tissue of the coffee plant, wherein the second inverted DNA sequence thereafter becomes inserted into the genome of the coffee plant.

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Fig 1: DEDUCED AMINO ACID SEQUENCE OF ACC SYNTHASE FROM *COFFEA ARABICA* (SEQ. ID. NO. 10)

Met Glu Phe Ser Leu Lys Asn Glu Gln Gln Gln Leu Leu Ser Lys  
1 5 10 15

5 Met Ala Thr Asn Asp Gly His Gly Glu Asn Ser Pro Tyr Phe Asp Gly  
20 25 30

10 Trp Lys Ala Tyr Asp Ser Asp Pro Tyr His Pro Thr Arg Asn Pro Asn  
35 40 45

Gly Val Ile Gln Met Gly Leu Ala Glu Asn Gln Leu Cys Phe Asp Leu  
50 55 60

15 Ile Glu Glu Trp Val Leu Asn Asn Pro Glu Ala Ser Ile Cys Thr Ala  
65 70 75

Glu Gly Ala Asn Lys Phe Met Glu Val Ala Ile Tyr Gln Asp Tyr His  
80 85 90 95

20 Gly Leu Pro Glu Phe Arg Asn Ala Val Ala Arg Phe Met Glu Lys Val  
100 105 110

Arg Gly Asp Arg Val Lys Phe Asp Pro Asn Arg Ile Val Met Ser Gly  
25 115 120 125

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Gly Ala Thr Gly Ala His Glu Thr Leu Ala Phe Cys Leu Ala Asp Pro  
130 135 140

Glu Asp Ala Phe Leu Val Pro Thr Pro Tyr Tyr Pro Gly Phe Asp Arg  
5 145 150 155

Asp Leu Arg Trp Arg Thr Gly Met Gln Leu Leu Pro Ile Val Cys Arg  
160 165 170 175

10 Ser Ser Asn Asp Phe Lys Val Thr Lys Glu Ser Met Glu Ala Ala Tyr  
180 185 190

Gln Lys Ala Gln Glu Ala Asn Ile Arg Val Lys Gly Phe Leu Leu Asn  
195 200 205

15 Asn Pro Ser Asn Pro Leu Gly Thr Val Leu Asp Arg Glu Thr Leu Ile  
210 215 220

Asp Ile Val Thr Phe Ile Asn Asp Lys Asn Ile His Leu Ile Cys Asp  
20 225 230 235

Glu Ile Tyr Ser Ala Thr Val Phe Ser Gln Pro Glu Phe Ile Ser Ile  
240 245 250 255

25 Ser Glu Ile Ile Glu His Asp Val Gln Cys Asn Arg Asp Leu Ile His  
260 265 270

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	Leu Val Tyr Ser Leu Ser Lys Asp Leu Gly Phe Pro Gly Phe Arg Val	
	275	280 285
5	Gly Ile Leu Tyr Ser Tyr Asn Asp Ala Val Val Ser Cys Ala Arg Lys	
	290	295 300
	Met Ser Ser Phe Gly Leu Val Ser Thr Gln Thr Gln His Leu Ile Ala	
	305	310 315
10	Ser Met Leu Ser Asp Glu Ala Phe Met Asp Lys Ile Ile Ser Thr Ser	
	320	325 330 335
	Ser Glu Arg Leu Ala Ala Arg His Gly Leu Phe Thr Arg Gly Leu Ala	
	340	345 350
15	Gln Val Gly Ile Gly Thr Leu Lys Ser Ser Ala Gly Leu Tyr Phe Trp	
	355	360 365
	Met Asp Leu Arg Arg Leu Leu Arg Glu Ser Thr Phe Glu Ala Glu Met	
	370	375 380
20	Glu Leu Trp Arg Ile Ile Ile His Glu Val Lys Leu Asn Val Ser Pro	
	385	390 395
25	Gly Leu Ser Phe His Cys Ser Glu Pro Gly Trp Phe Arg Val Cys Phe	
	400	405 410 415

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Ala Asn Met Asp Asp Glu Ser Val Arg Val Ala Leu Arg Arg Ile His  
420 425 430

Lys Phe Val Leu Val Gln Gly Lys Ala Thr Glu Pro Thr Thr Pro Lys  
5 435 440 445

Ser Arg Cys Gly Ser Ser Lys Leu Gln Leu Ser Leu Ser Phe Arg Arg  
450 455 460

10 Leu Asp Glu Arg Val Met Gly Ser His Met Met Ser Pro His Ser Pro  
465 470 475

Met Ala Ser Pro Leu Val Arg Ala Thr  
480 485

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Fig 2.: Coffee fruit-expressed ACC synthase gene sequence. (SEQ. ID. NO. 11)

	GTAACTCTCTT CTAAAATCAA CCATTCTCTT CATTCTTCAC TTGACAAGGC CACTGCATTTC	60
	TTCAATTCCTTT CTTGATATAT AGCCATTTTT TTCATTCTTT CTTGATATAT AGCCATTTTT	120
5	TTCAATTCCTTT CTTCAATTCAT TGTCTGGAGA AGTTGGTTGA GTTTCTTGA AAATTCAAGC	180
	AAAACA ATG GAG TTC AGT TTG AAA AAC GAA CAA CAA CTC TTG TCG AAG	231
	ATG GCA ACC AAC GAT GGA CAT GGC GAA AAC TCG CCT TAT TTT GAT GGT	279
10	TGG AAG GCA TAT GAT AGT GAT CCT TAC CAT CCC ACC AGA AAT CCT AAT	327
	GGT GTT ATA CAG ATG GGA CTC GCA GAA AAT CAG TTA TGC TTT GAT TTG	375
15	ATC GAG GAA TGG GTT CTG AAC AAT CCA GAG GCT TCC ATT TGC ACA GCA	423
	GAA GGA GCG AAC AAA TTC ATG GAA GTT GCT ATC TAT CAA GAT TAT CAT	471
	GGC TTG CCA GAG TTC AGA AAT GCT GTA GCA AGG TTC ATG GAG AAG GTG	519
20	AGA GGT GAC AGA GTC AAG TTC GAT CCC AAC CGC ATT GTG ATG AGT GGT	567
	GGG GCA ACC GGA GCT CAT GAA ACT CTG GCC TTC TGT TTA GCT GAC CCT	615
25	GAA GAT GCG TTT TTG GTA CCC ACA CCA TAT TAT CCA GGA TTT GAT CGG	663
	GAT TTG AGG TGG CGA ACA GGG ATG CAA CTT CTT CCA ATT GTT TGT CGC	711
	AGC TCC AAT GAT TTT AAG GTC ACT AAA GAA TCC ATG GAA GCT GCT TAT	759
30	CAG AAA GCT CAA GAA GCC AAC ATC AGA GTA AAG GGG TTC CTC TTA AAT	807

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	AAT CCA TCA AAT CCA TTG GGA ACT GTT CTT GAC AGG GAA ACT TTG ATT	855
	GAT ATA GTC ACA TTC ATC AAT GAC AAA AAT ATC CAC TTG ATT TGT GAT	903
5	GAG ATA TAT TCT GCC ACC GTC TTC AGC CAG CCC GAA TTC ATC AGC ATC	951
	TCT GAA ATA ATT GAG CAT GAT GTT CAA TGC AAC CGT GAT CTC ATA CAT	999
	CTT GTG TAT AGC CTG TCC AAG GAC TTG GGC TTC CCT GGA TTC AGA GTT	1047
10	GGC ATT TTG TAT TCA TAT AAT GAC GCT GTT GTC AGC TGT GCT AGA AAA	1095
	ATG TCG AGT TTC GGC CTT GTT TCA ACA CAA ACT CAG CAT CTG ATT GCA	1143
15	TCA ATG TTA TCG GAC GAA GCA TTT ATG GAC AAA ATC ATT TCC ACG AGC	1191
	TCA GAG AGA TTA GCT GCA AGG CAT GGT CTT TTC ACA AGA GGA CTT GCT	1239
	CAA GTA GGC ATT GGC ACC TTA AAA AGC AGT GCG GGC CTT TAT TTC TGG	1287
20	ATG GAC TTA AGG AGA CTC CTC AGG GAG TCC ACA TTT GAG GCA GAA ATG	1335
	GAA CTT TGG AGG ATC ATA ATA CAT GAA GTC AAG CTC AAT GTT TCA CCA	1383
25	GGC TTA TCT TTC CAT TGC TCA GAA CCA GGA TGG TTC AGA GTT TGC TTT	1431
	GCC AAC ATG GAC GAC GAA AGT GTG AGA GTT GCT CTC AGA AGA ATC CAC	1479
	AAA TTT GTG CTT GTT CAG GGC AAG GCA ACA GAG CCA ACA ACT CCA AAG	1527
30	AGT CGC TGC GGA AGC AGC AAA CTT CAA CTC AGC TTA TCT TTC CGC AGA	1575

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TTG GAC GAA AGG GTG ATG GGA TCG CAT ATG ATG TCC CCT CAC TCC CCG 1623

ATG GCT TCA CCT TTG GTT CGG GCT ACA TAAATCATTT CTTGATCAGA TCATATAGCA 1680

5 AAGATTCCCTG AGTAAATACT CGAAACCCTT TCTGGATAAC TGAAAAGAGA GTTGTGATT 1740

CTTTGCTGTA TCATACAAAC ACGTTACAGG CATTTTTTGG CCATCTGATG CGTGCAAATT 1800

GCAACAAATG CTTTTATTAT TGTCAATATC ATTTGTGTAC CTTGGTTTTT CTTGCCCTTC 1860

AGTCCTCCTT GTTTTTTGTT TCTTTGTTAT TATTTTCTTC CAGTTGATCA GTTAAACGAA 1920

GGAAGCTCAA TTGTTTCAAG CTATTAGTAA CAGATCATTT TGTAATAGCA ATAGTTTCAG 1980

10 GATTCTGAAA TGAAAGTTTA TCATTTTTC ATCATTTTAA AAAAAAAAAA AAAAAAAAAA 2040

Note: The coding portion of this sequence is shown by grouping the bases as codons.



**Fig. 3: DEDUCED PROTEIN SEQUENCE OF THE COFFEE FRUIT-EXPRESSED ACC OXIDASE cDNA (SEQ. ID. NO. 12)**

09:45:36

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Phe Ala Leu Gln Leu Glu Lys Leu Ala Glu Leu Leu Leu Asp Leu Leu  
120 125 130

Cys Glu Asn Leu Gly Leu Glu Lys Gly Tyr Leu Lys Lys Ala Phe Tyr  
5 135 140 145

Gly Thr Lys Gly Pro Thr Phe Gly Thr Lys Val Ser Asn Tyr Pro Pro  
150 155 160

10 Cys Pro Arg Pro Glu Leu Ile Lys Gly Leu Arg Ala His Thr Asp Ala  
165 170 175 180

Gly Gly Ile Ile Leu Leu Phe Gln Asp Asp Lys Val Ser Gly Leu Gln  
185 190 195

15 Leu Leu Lys Asp Gly Glu Trp Val Asp Val Pro Pro Met Arg His Ser  
200 205 210

Ile Val Ile Asn Ile Gly Asp Gln Leu Glu Val Ile Thr Asn Gly Lys  
20 215 220 225

Tyr Lys Ser Val Met His Arg Val Ile Ala Gln Pro Asp Gly Asn Arg  
230 235 240

25 Met Ser Leu Ala Ser Phe Tyr Asn Pro Gly Ser Asp Ala Val Ile Tyr  
245 250 255 260

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Pro Ala Pro Ala Leu Val Glu Lys Glu Ala Glu Asp Lys Gln Ile Tyr

265

270

275

Pro Lys Phe Val Phe Glu Asp Tyr Met Lys Leu Tyr Ala Gly Leu Lys

5

280

285

290

Phe Gln Ala Lys Glu Pro Arg Phe Glu Ala Met Lys Ala Val Glu Ser

295

300

305

10 Thr Val Asn Leu Gly Pro Ile Ala Thr Val

310

315

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Fig 4: DNA SEQUENCE OF THE COFFEE FRUIT-EXPRESSED ACC OXIDASE CDNA.  
(SEQ. ID. NO. 13)

	TGTAAACGAA GCATAAGCAC AAGCAAACAC AAAC TAGAAA GAGAG ATG GCT ACA TTC	57
5	CCC CTA ATC GAC ATG GAG AAG CTT GAC GGT GAA GAG AGG GCT GCC ACT	105
	ATG GGA GTC ATA AAA GAT GCT TGT GAA AGC TGG GGC TTC TTT GAG GTG	153
10	TTG AAT CAT GGG ATA TCT AAT GAG CTC ATG GAC ACA GTG GAG AGG CTA	201
	ACA AAG GAG CAT TAC AAG AAA TGT ATG GAA CTA AAG TTC AAG GAA ATG	249
	GTG GAG AGC AAG GAA TTG GAA GCT GTT CAG ACT GAG ATC AAT GAT TTG	297
15	GAC TGG GAA AGT ACC TTC TTC TTG CGC CAT CTT CCT GTT TCC AAC ATC	345
	TCA GAA GTC CCT GAT CTT GAT GAT GAA TAC AGA AAG GTT ATG AAG GAA	393
20	TTT GCG TTG CAA CTT GAG AAA CTA GCA GAG CTC CTG TTG GAC TTG CTA	441
	TGC GAG AAC CTT GGC CTA GAG AAA GGC TAT CTG AAG AAA GCC TTC TAT	489
	GGC ACC AAA GGA CCA ACC TTT GGC ACC AAA GTC AGC AAT TAC CCT CCA	537
25	TGC CCT CGT CCA GAA CTG ATC AAG GGC CTC CGG GCA CAC ACC GAT GCC	585
	GGC GGC ATC ATC CTG CTG TTC CAG GAT GAC AAG GTC AGC GGT CTC CAG	633
30	CTC CTC AAG GAT GGT GAA TGG GTG GAT GTT CCG CCT ATG CGC CAC TCC	681
	ATT GTA ATC AAC ATC GGC GAC CAA CTT GAG GTA ATC ACA AAT GGA AAA	729

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	TAC AAG AGT GTG ATG CAC CGG GTG ATA GCT CAA CCA GAT GGG AAC AGA	777
	ATG TCA CTA GCA TCA TTC TAC AAT CCA GGA AGT GAT GCA GTG ATC TAT	825
5	CCA GCA CCG GCA TTG GTT GAG AAA GAG GCA GAG GAC AAG CAG ATA TAT	873
	CCC AAG TTT GTG TTC GAG GAC TAC ATG AAG CTC TAT GCT GGC CTT AAG	921
	TTC CAA GCT AAA GAG CCC AGG TTT GAA GCC ATG AAG GCC GTG GAA AGC	969
10	ACC GTA AAC TTG GGT CCA ATC GCA ACT GTT TGAGATAATA CACGCTTTGA	1019
	TCTGCTGCTG TCTTATAATG CGCGTTTGCG TAATCATATC CTAGCATAGT ATATCTGAGA	1079
15	TCTGAGTCTG TATTGTGGTG TGAGTTTGGT TTAGCCCCCTT GTTAATGCTT GGATTGGACT	1139
	AGTTAAATGT GGAGCTGGTT TGTTAGATAA GATAGTCTTG CCAGGATCTT TGAGTAAATA	1199
	TGATTCTGCG GAAGTCTGCG GTGAATGATA ACGTGTAAG CAATCCGAAA GTTACCTTTC	1259
20	TGGGGCTTTG TCATATGCAA TGGAGAAGGA ATCTTCCAAA AAAAAAAAAA AAAAAAAAAA	1319
	A	1320
25	Note: The coding portion of this sequence is shown by grouping the bases as codons.	

## INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/US 97/14184

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/52 C12N15/53 C12N15/11 C12N15/82 C12N9/02  
C12N9/88 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 19103 A (DNA PLANT TECHN CORP) 27 June 1996  * see esp. pp.16-19, pp.23/24, ex.9,10 *	13-21, 28,29, 31,32
A	WO 91 01375 A (ICI PLC) 7 February 1991 see the whole document	1-33
A	WO 92 04456 A (US OF AMERICA REPRESENTED BY T) 19 March 1992 see the whole document	1-33
A	WO 96 07742 A (ASGROW SEED CO ;BOESHORE MAURY L (US); DENG ROSALINE Z (US); CARNE) 14 March 1996 see the whole document	1-33

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

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Date of the actual completion of the international search

2 December 1997

Date of mailing of the international search report

19/12/1997

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# INTERNATIONAL SEARCH REPORT

Inter:      nal Application No  
PCT/US 97/14184

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 96 21027 A (ASGROW SEED CO ;BOESHORE MAURY L (US); DENG ROSALINE Z (US); CARNE) 11 July 1996 see the whole document -----</p>	1-33

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